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Prospects of vaccines for medically important fungi

This is the first of a series of reviews that will appear periodically over the next two years that detail the basic science progress in the quest to achieve vaccines for several of the medically important fungi.

Review

A vaccine against coccidioidomycosis is justified and attainable

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Coccidioides is a fungal pathogen of humans which can cause a life-threatening respiratory disease in immunocompetent individuals. Recurrent epidemics of coccidioidal infections in Southwestern United States has raised the specter of awareness of this soil-borne microbe, particularly among residents of Arizona and Southern California, and has galvanized research efforts to develop a human vaccine against coccidioidomycosis. In this review, we discuss the rationale for such a vaccine, examine the features of host innate and acquired immune response to *Coccidioides* infection, describe strategies used to identify and evaluate vaccine candidates, and provide an update on progress toward development of a vaccine against this endemic pathogen.

Introduction

Coccidioides is the etiologic agent of a human respiratory infection known as coccidioidomycosis or San Joaquin Valley fever [1]. The clinical manifestations of this disease range from an influenza-like illness that resolves spontaneously, to a hematogenously and lymphogenously disseminated mycosis in which the pathogen can migrate from the lungs to the skin, bone, meninges and, in some cases, even to the peritoneum, heart and multiple other body organs [2],[3]. About half of the *Coccidioides*-infected individuals experience only mild discomfort and do not seek medical intervention. On the other hand, these same individuals could be at risk of reactivation of coccidioidal infection later in life if they contract an immunocompromising disease or undergo organ transplantation with elective immunosuppression 4–8. Reactivation of *Coccidioides* infection may occur in pregnant women during their third trimester [9], when it has been shown that the risk of dissemination of the fungal pathogen is highest [10]. An intriguing case of coccidioidomycosis has been reported in which a concomitant bacterial and viral infection apparently predisposed the individual to reactivation of the fungal infection [11].

Approximately half of the presumably immunocompetent individuals exposed to *Coccidioides* develop symptomatic infection, which manifests one to four weeks after inhalation of the fungal spores. The disease at this stage is an atypical pneumonia, characterized by such conditions as pleuritic chest pain, cough, fever, malaise, rashes, mild sore throat, headache, arthralgia, myalgia, or anorexia [12]. Most infected individuals recover during the subsequent few weeks to several months, while approximately 5 to 10 percent show evidence of active disease based on positive results of

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serology and chest radiographs. Detection of rising titers of patient immunoglobulin G (IgG) antibody to *Coccidioides* antigen provides a definitive diagnosis of active coccidioidomycosis. Microscopic examinations of sputum or bronchoscopy specimens are used for diagnosis of the pulmonary form of the disease. On rare occasions, particularly in patients with acquired immunodeficiency syndrome (AIDS), the results of bronchoscopic and serologic tests for *Coccidioides* infection are negative or inconclusive, making differential diagnosis of the disease problematic [13]. Patients who present with any of the primary symptoms of pulmonary infection described above, as well as positive chest radiographs and enlarged lymph nodes composed of caseating granulomas, are not necessarily infected with *Coccidioides*. There is a close similarity between the clinical presentations of coccidioidomycosis and tuberculosis, including caseating granulomas which are observed in both pulmonary diseases [14]. The most reliable evidence of coccidioidal infection is positive identification of the pathogen in cultures of sputum, bronchoalveolar lavage fluids (BALFs) or tissue biopsies obtained from patients with active disease.

Antifungal drug treatment of persons with newly diagnosed coccidioidomycosis may hasten symptom resolution, but physicians decide on a case-by-case basis whether a patient's circumstances warrant early therapy [15]. Previous studies of patients treated with fluconazole indicated that the relapse rate following therapy is high (approx. 37%), and treatment trials with higher doses of azoles were recommended [16]. Currently, an aggressive therapeutic protocol (e.g., 400 mg/d for ketoconazole, 400 mg/d for fluconazole, or 200 mg twice daily for itraconazole) is recommended if symptoms persist for more than 2 months. Drug therapy is commonly maintained for at least 3 to 6 months. Coccidioidomycosis contracted by patients infected with HIV often requires life-long antifungal medication to suppress reactivation of the fungal disease [17]. It has been suggested that initial treatment of the immunosuppressed host should always be with amphotericin B rather than an azole [18]. Azole treatment may be used as a follow-up for patients who respond quickly to amphotericin B. The total yearly cost to the patient, based on a retrospective study of 536 cases conducted between 1991 and 1993 at the Kern Medical Center in Bakersfield, California, was estimated at $8,096 [19]. The breakdown of expenses was as follows: 63% attributed to hospitalization, 18% to clinic visits, 12% to lost wages, and 7% to the cost of drug treatment. Non-disseminated cases averaged $5,400/year, while patients who contracted the disseminated form of the disease faced an average cost of $48,000. Current costs incurred by patients with severe coccidioidal infection can climb to $300,000, and a conservative estimate of the annual health care expenses for treatment of patients with coccidioidomycosis in the U.S. is $60 million (www.valleyfever.com). Vaccination against *Coccidioides* infection has been argued to be a cost-effective intervention [20] and, on this basis alone, is worthy of serious consideration. In this review, we examine the rationale and strategies used for development of such a vaccine, describe methods employed in the evaluation of vaccine candidates, and discuss the research progress of a consortium of investigators committed to the generation of a human vaccine against coccidioidomycosis.

Natural reservoirs and host range

Coccidioides typically inhabits desert and semi-arid soils where it grows as a filamentous saprobe. Fertile hyphae of this vegetative phase give rise to dry, asexual, air-dispersed spores (arthroconidia), which are the infectious propagules of the pathogen [21]. A sexual state of *Coccidioides* has never been observed, although evidence has been presented that recombination ('cryptic sex') between isolates does occur in nature [22]. Coccidioidomycosis is endemic to desert regions of Southern California, Arizona, Nevada, New Mexico and West Texas, as well as parts of Mexico, Central America and South America [23,24]. Isolation of *Coccidioides* from the environment has been difficult. The organism was first cultured from soil in 1932 [25,26], but most new isolates are obtained directly from clinical specimens. Polymerase chain reaction (PCR) was used to amplify a *Coccidioides*-specific internal transcribed spacer (ITS) of ribosomal DNA, and employed as a probe to search for environmental isolates [27]. However, in spite of the high sensitivity and specificity of this assay, only 4 isolates were obtained from a screen of 720 separate soil samples collected from endemic areas in California. A possible explanation for this is that the fungus does not survive in the upper layers of desert soil during the hot summer months, but instead resides in cooler, subsurface zones which are supplied adequate nutrients for its growth. In fact, studies have shown that greater numbers of the organism are found several inches below the surface of the soil where temperatures are significantly cooler [28] and nutrients are more abundant. Large reservoirs of bioavailable nitrogen (up to approx. 10^4 kilograms of nitrogen per hectare, as nitrate) have been shown to accumulate in the subsurface soils of desert regions and arid shrublands which are endemic for coccidioidomy-

cosis [29]. Burrowing rodents (kangaroo rats, pocket mice) and armadillos have been suggested to play roles in the harboring and spread of the pathogen in endemic regions of North and South America [23,30] Coccidioidomycosis is frequently reported in domesticated dogs that live outdoors in the Southwest [31,32]. This raises the possibility that wild dogs, desert foxes and coyotes may also contribute to the ecology of this environmental pathogen. Probably all mammals residing in the endemic regions are at risk of coccidioidal infection [33,34]. Death and subsequent decay of *Coccidioides*-infected animals under natural conditions culminates in conversion of the parasitic phase of the fungus into its mycelial form, thus recharging the soil with mycelia that yield profuse 'arthroconidial blooms.' Detection of endosporulating spherules of *Coccidioides* in lesions isolated from the lungs of a Sonoran Gopher snake [35] illustrates that the pathogen is not restricted to homoiotherms. Casadevall and Pirofski [36] have proposed that *Coccidioides* and other pathogenic, soil fungi that cause systemic infections in humans may have alternative invertebrate and protozoan hosts. The authors have argued that the virulence of these soil-inhabiting microbes in mammalian hosts may originate from environmental selective pressures imposed by amoeboid and nematode predators. Foraging activities of these microorganisms in arid soils would be restricted to brief seasons of rainfall. Outbreaks of human infections with *Coccidioides* typically follow the rainy seasons. It has been proposed (A. Casadevall, pers. comm.) that protozoa such as amoebae could engulf *Coccidioides* cells during periods of foraging, encyst during arid periods, and then germinate again in moist soils. This cycle of selection for survival of the fungus in phagocytic cells could translate to its survival in human macrophages. These are intriguing ideas which require further study, first to determine whether such alternative hosts for *Coccidioides* actually occur in nature and, if so, whether the proposed selective pressure contributes to 'fungal fitness' and virulence in humans.

Genetic diversity of the pathogen

Fisher and coworkers [37] have proposed that North American populations of *Coccidioides* are genetically diverse, based on the observation that molecular markers reveal differentiation among isolates from California on the one hand, and Arizona and Texas on the other [38]. It was suggested that North American isolates belong to two major clades, previously designated as the Californian (CA) and non-Californian (non-CA) phylogenetic species [39,40]. Isolates from South America, on the other hand, were shown to be genetically less diverse and suggested to have arisen from a single population centered in Texas. More recent studies, which involved analyses of nucleotide sequence diversity between microsatellite loci, have confirmed the molecular distinction between the CA and non-CA isolates [41], and led to formal revision of the generic concept of *Coccidioides* to include two separate species [42]. *C. posadasii* is a newly described taxon which accommodates the non-CA clade and is widespread throughout most of the endemic regions of the U.S., Mexico and Central and South America. *C. immitis* includes the CA clade that is found primarily in the San Joaquin Valley of California. The authors demonstrated that *C. posadasii* can be distinguished from *C. immitis* by numerous DNA polymorphisms, and that microsatellite loci can be used as diagnostic markers for this species. Although *C. posadasii* isolates appear to grow slower on high salt media than *C. immitis*, the morphologies of the saprobic and parasitic phases of these two species appear to be identical. However, their genetic distinctions may be translated into differences in amino acid composition, antigenicity and virulence [42]. On the basis of this supposition, two separate genome-sequencing projects have been initiated for *Coccidioides*. The genome of *C. posadasii* is being sequenced at The Institute for Genomic Research (www.tigr.org); while nucleotide sequence analysis of *C. immitis* has begun at the Whitehead Institute/MIT Center for Genome Research as part of an ambitious, multi-genus fungal genome initiative (www.genome.wi.mit.edu/annotation/fungi/fgi/). The genomic sequence of *C. posadasii* is essentially complete, and annotation of the database is underway.

In vitro/in vivo morphogenesis and methods of identification of *Coccidioides*

The morphology of the saprobic phase of *Coccidioides* is indistinct from several closely related ascomycetous members of the Onygenales [43–45]. Identification of the pathogen in the past has been dependent upon both its ability to infect mice, and the differentiation of arthroconidia into multicellular, parasitic forms (endosporulating spherules) in vitro and in vivo. Molecular methods of *Coccidioides* culture identification and detection in infected host tissue are now more widely used in clinical settings. These include the application of commercially available chemiluminescent DNA probe (Accuprobe) assays [Gen-Probe Inc., San Diego, Calif.; [46]], in situ hybridization of DNA probes with tissue sections [47], and oligonucleotide probes for PCR amplification of pathogen-specific genes [48–50]. The histopathology of *Coccidioides* infections is character-

ized by the presence of well-defined spherules in various stages of development, which to the trained observer is a diagnostic feature of coccidioidomycosis. However, human *Coccidioides* infections occasionally reveal hyphal elements in surgically excised and resectioned cavitary and granulomatous pulmonary lesions in human lungs [51], which can complicate the diagnosis of this disease. Mycelial forms are also observed in cutaneous lesions and in the central nervous system of *Coccidioides*-infected patients. Hyphal growth in these latter cases has been frequently associated with plastic implants (e.g., shunts or reservoirs) used to treat infection of the brain or cerebrospinal fluid [52]. Typically, once arthroconidia are inhaled they transform into spherules in terminal bronchi and begin to endosporulate. This sequence of morphogenetic events can be reproduced in vitro by growth of the organism in a defined glucose-salts medium purged with 20% CO_2/80% air [53] (Fig. 1).

Epidemiology of coccidioidomycosis

Coccidioidal infection of humans most commonly occurs by inhalation of arthroconidia [54], although percutaneous inoculation is also possible. It is not known how many propagules produced by the soil-borne, saprobic phase must be inhaled by humans to establish symptomatic disease [55]. However, intranasal inoculation with approximately 10 arthroconidia is sufficient to kill BALB/c mice within 21 days post-challenge [56]. Person-to-person transmission of *Coccidioides* is not known to occur, except in rare cases of maternal-fetal transfer of infected tissue [57]. Epidemiological studies of coccidioidomycosis have revealed that the interaction of environmental rather than genetic factors has a major impact on recurrent epidemics of this disease [58,59]. Important environmental variables include the duration of droughts preceding epidemics, and the timing and amounts of rainfall following arid seasons. These factors contribute significantly to the extent and density of the fungal blooms in soil reservoirs. Because soil disturbance and extensive outdoor activity enhance the chances of infection, coccidioidomycosis is considered an occupational disease amongst agricultural and construction workers [28]. Coccidioidomycosis is also a geographically limited disease, but the size of the resident population in the endemic regions and the number of visitors attracted to these parts of the nation are rapidly growing. The population of the major endemic areas of Southern Arizona has increased by a factor of 10 over the past 50 years, and by a factor of 4–5 in the Central Valley of California and West Texas. There are now many more people at risk of coccidioidal infection than there were 50 years ago, particularly because of the surge in the number of 'serologically virgin' immigrants [28] from the North and East. About 8 million people live in the most highly endemic region of the Southwestern United States, and millions more in the neighboring regions of coastal Southern California. Early reports estimated that 40% of persons infected with *Coccidioides* develop symptomatic disease. Most of these individuals (85%) present with a mild influenza-like illness, while 8% may develop severe pulmonary disease requiring hospitalization, and 7% develop the disseminated extrapulmonary form of the mycosis [60]. Risk factors for severe pulmonary and disseminated disease include African-American, Filipino or Asian race, pregnancy, immunocompromising conditions (e.g., organ transplantation, AIDS), diabetes, smoking and older age (65 years+) [8,61–65]. Estimates of the number of individuals in endemic regions of Mexico, Central and South America who may be subject to primary infection and are likely to develop

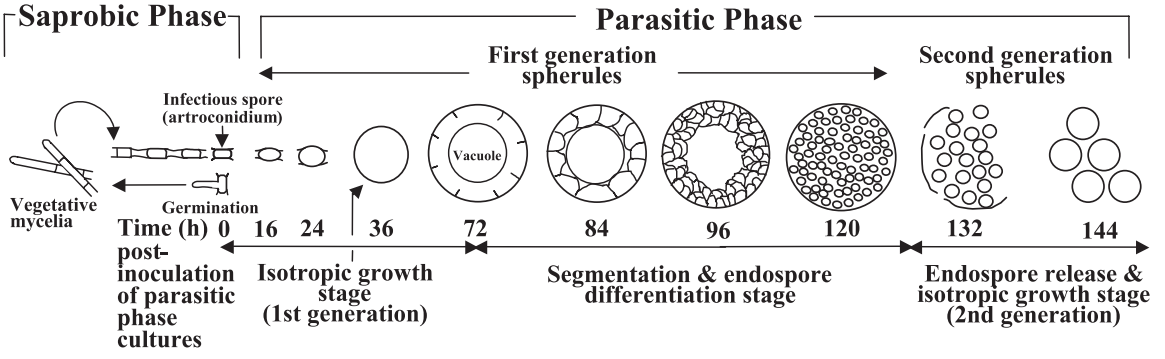

Fig. 1 Diagrammatic representation of the saprobic and parasitic cycles of *Coccidioides posadasii* (reproduced with permission from Taylor and Francis and the American Society for Microbiology; taken from references190 (Infect. Immun) and 230 (Med. Mycol).

severe disease are further complicated by environmental, socioeconomic, and substandard health conditions [66]. New estimates of the percentage of people in endemic regions who contract symptomatic coccidioidomycosis are based on data derived from studies of recent outbreaks of coccidioidal infection and have been shown to be greater than 50%. For example, 27 of 35 (77%) volunteers from Pennsylvania who traveled to Hermosillo, Mexico to help construct a church developed an influenza-like illness within two weeks after returning home and, based on clinical evaluations, were all suspected to have contracted coccidioidomycosis [60]. A similar outbreak of coccidioidal infection was reported amongst a 126-member church group that had traveled to Tecate, Mexico to assist in construction of an orphanage [67]. These cases underscore the risk of coccidioidal infection of immunocompetent individuals who engage in construction or other activities that raise dust in endemic regions [68]. An outbreak of symptomatic coccidioidomycosis was reported in 10 of 18 workers (56%) at an archeological site in Northeastern Utah [69]. This represents the first occurrence of *Coccidioides* infection of humans in this part of North America. Sporadic cases of coccidioidomycosis are regularly reported in regions where the disease is not endemic (e.g., New York) [70]. Acquisition of the disease in these cases is almost always confirmed to be travel-related [68,71–73].

The most dramatic increase in the number of reported cases of coccidioidomycosis has occurred in Arizona [74–76]. During 1997, laboratory reporting of coccidioidal infections became mandatory in Arizona, after which a significant increase in the number of cases was noted. In 2001, a total of 2,203 cases were reported (rate of 43 per 100,000 population), compared to 1,551 cases in 1998 (rate of 33). Persons aged ≥ 65 years and individuals with HIV infection had the highest incidence, although symptomatic infections in all age groups increased [76]. Coccidioidomycosis is now a nationally reportable disease at the southwest regional level through the National Electronic Telecommunication System for Surveillance (NETSS). A case definition that required laboratory confirmation of coccidioidal infection was adopted. The laboratory criteria for diagnosis are cultural, histopathologic, or molecular evidence of the presence of *Coccidioides* spp.; a positive serologic test for coccidioidal antibodies in serum or cerebrospinal fluid based on 1) detection of coccidioidal IgM by immunodiffusion, enzyme immunoassay (EIA), latex agglutination, or tube precipitin reaction, or 2) detection of a rising titer of IgM or complement fixation (CF) antibody (IgG) by immunodiffusion, EIA or the CF test. Positive laboratory tests combined with coccidioidal skin test conversion from negative to positive after onset of clinical signs and symptoms [75] support the diagnosis of coccidioidal infection.

Galgiani [15] has pointed out that, although extensive epidemiological studies of coccidioidal infections were performed in the U.S. during the 1940s and 50s [77,78], nearly a half-century later *Coccidioides* has been rediscovered as an emerging pathogen [79]. Several factors have contributed to the current high profile of this endemic disease. As previously mentioned, a continuing increase of the resident population in the Southwestern U.S. and the rising number of visitors to the 'Sunbelt' has substantially elevated the risk of human infection. Arizona is the third fastest growing state in the U.S. and attracts large numbers of elderly citizens, particularly from the northern region of the country. In almost all cases, these individuals have not been previously exposed to the pathogen, and are at high risk of contracting symptomatic coccidioidomycosis. The increased incidence of immunocompromised persons in communities at large, including patients with AIDS and cancer, and individuals who undergo organ transplantation, has created an expanded host population for this opportunistic fungus. The endemic regions of coccidioidomycosis in the U.S. include several major military installations, and recent studies have emphasized the significance of *Coccidioides* as a threat to military personnel who train in southwest desert areas [80]. A study conducted on 1,438 troops stationed at a military site in Southern California showed that the skin test conversion rate from negative to positive reactivity with *Coccidioides* antigen was 25.4% over a six-to-eight month period after new recruits arrived on the base [81]. The results of this study exemplify what can happen even when a young, presumably immunocompetent but naïve population moves into an endemic area. The fact that this fungus is a primary pathogen is well documented by a report of coccidioidal infection in 10 of 22 members of a deployment of Navy Seals on military maneuvers in the San Joaquin Valley. All case patients were symptomatic, and 50% had abnormal chest radiographs [82].

The high virulence of *Coccidioides*, ease of infection by inhalation of dry, air-dispersed spores, and biogeographic range in heavily populated regions of North America (including military bases) has raised fears of the biohazardous nature and bioweaponizing potential of this human pathogen [83]. It has been argued, however, that *Coccidioides* is an unlikely choice as a biological weapon [84]. Nevertheless, the Centers for Disease Control now mandate that research involving live cultures of the infectious phase of *Coccidioides* be

conducted under the containment conditions of a biological safety level 3 (BSL3) laboratory. *Coccidioides* spp. is the only fungal pathogen on the 'select agent' list, which includes viruses, bacteria and toxins considered to be potential bioweapons (www.nih.gov/od/ors/ds/pubs/appendxa.html). Research involving these agents is currently regulated under the US Public Health Security and Bioterrorism Preparedness and Response Act of 2002.

In January 2003, a National Institute of Allergy and Infectious Disease (NIAID)-appointed Blue Ribbon Panel examined various issues related to "Biodefense and Its Implications for Biomedical Research" and published its recommendations for the revised NIAID categories B and C Priority Pathogen Lists (www.niaid.nih.gov/biodefense/research/categorybandc.pdf). It was recommended that *Coccidioides* be added to the category B list, which would mean that investigators working with this pathogen would be eligible for biodefense research funds that are currently available for studies of other microbes on the A, B, and C lists. At present, however, investigations of *Coccidioides* and coccidioidomycosis remain ineligible for these research funds, even though scientists working with the pathogen are subject to the same time-consuming and costly regulations as those investigating select agents that do qualify for biodefense research grants. We have argued that *Coccidioides* should be either added to the NIAID category 'B' or 'C' list of priority pathogens, or deleted from the lists of select agents.

Protection against coccidioidomycosis is feasible

A compelling argument for the feasibility of a vaccine against coccidioidomycosis is that natural, human infection with *Coccidioides* results in lifelong immunity to the organism [1,85]. Several early studies of experimental coccidioidomycosis in animal models showed that immunization with an attenuated strain of the pathogen protected against a lethal, intranasal infection of the host [86,87]. The mouse is the most extensively used animal model for studies of coccidioidal infection [88], and differences in susceptibility to disease have been observed between murine strains (BALB/c > C57BL/6 > DBA/2) [89]. BALB/c mice are the most susceptible to disseminated coccidioidomycosis following intranasal challenge with arthroconidia, and this strain has been used as a stringent test of the protective efficacy of experimental vaccination protocols. Although immunization of BALB/c mice with the original attenuated strains of *Coccidioides* prolonged the survival of the animals against a lethal challenge, both the vaccinating organism and pathogen apparently persisted in the host. In addition, avirulence proved to be an unstable condition in these early experiments, which led to the abandonment of live vaccines against the disease [85]. However, live attenuated vaccines against other infectious diseases, such as the Bacille Calmette-Guérin (BCG) vaccine against tuberculosis, have revealed some degree of success and are still widely used [90–92]. With the advent of recombinant DNA technology, attenuated strains of microbial pathogens have been generated by specific gene deletion strategies, and this has provided both a clear understanding of the mechanism by which pathogenicity is inactivated, and confidence that the mutated strain will not revert to the virulent form. A mutant strain of *C. posadasii* which has been shown to be avirulent in mice was generated in our laboratory by the deletion of a single chitin synthase gene (*CHS5*) [93]. The null mutant (Δ*chs5*) showed atypical morphology and retarded growth in vitro, and failed to develop into the parasitic phase in vivo. BALB/c mice were immunized subcutaneously with the live Δ*chs5* strain together with incomplete Freund's adjuvant (IFA), and then challenged intranasally with a lethal inoculum of arthroconidia derived from the parental strain of *C. posadasii*. The adjuvant was included during the vaccination protocol to help generate a depot of immunogen. However, replacement of IFA with PBS had no apparent effect on protective immunity. All immunized animals survived beyond 45 days post-challenge, compared to none of the control mice (Fig. 2). In addition, 11 of the 12 Δ*chs5*-immunized survivors showed clearance of viable fungal elements (including the vaccination strain) from the lungs, liver and spleen. The U.S. Department of Health and Human Services designated the genetically engineered Δ*chs5* isolate of *C. posadasii* as an avirulent strain of a select agent. This avirulent strain does not pose a threat to public health and safety and can be used for the development of diagnostics, vaccines, and therapeutics (www.cdc.gov/od/sap/exclusion.htm). Although it is unlikely that a genetically engineered, avirulent strain of *Coccidioides* would be acceptable as a human vaccine, these results demonstrate the possibility of potent and durable protection against coccidioidal infection in a highly susceptible, immunocompetent host. The results of this study also demonstrate that host exposure to saprobic phase antigens stimulates immune responses that protect against the parasitic phase of the fungus. Spherules and endospores are the dominant morphotypes in infected tissue. However, it would seem that common protective

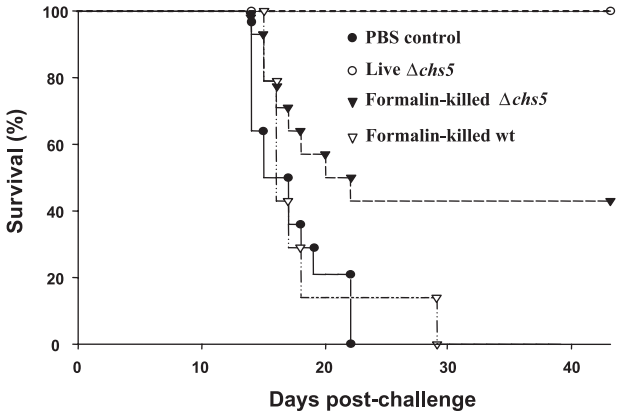

Fig. 2 BALB/c mice immunized with a live, avirulent strain of *C. posadasii* (○; Δ*chs5*) showed 100% protection against a lethal challenge of the parental strain (C735). Survival plots of BALB/c mice immunized with the formalin-killed Δ*chs5* (▼) or the formalin-killed wild type (wt) strain (▽) and challenged by the same route are compared to the survival of non-immunized mice (●). In each case, female mice (18–20 gm; 12 per group) were immunized subcutaneously with hyphal fragments (250–750 colony-forming units [CFUs] suspended in 100 µl of 50% [v/v] IFA in PBS). Immunization was performed twice, two weeks apart, using the same immunogen. The mice were challenged with 100 viable arthroconidia by the intranasal route 4 weeks after the second immunization (boost). In a separate experiment (results not shown), immunization with the live, avirulent Δ*chs5* stain was performed as above but in the absence of IFA. All mice survived beyond 45 days post-challenge, and the pathogen was cleared from their lungs, liver, and spleen.

antigens are expressed by hyphal elements and parasitic cells.

An experimental, formalin-killed spherule (FKS) vaccine has also been shown to protect mice against disseminated coccidioidal infection, indicating that a non-viable immunization strategy is feasible [94]. Between 1980 and 1985, a double-blinded human trial was conducted using the FKS vaccine versus a placebo [95]. The study involved almost 3000 volunteers, but only a minority of the vaccinated individuals became skin test-positive to *Coccidioides*. There was no difference in the number of cases of coccidioidomycosis or the severity of disease in the FKS-vaccinated group compared to the placebo group. One possible explanation for the ineffectiveness of this vaccine is that relatively small numbers of killed, parasitic cells could be injected into humans without unacceptable local side effects of pain and swelling. In vitro studies with FKS were performed on human peripheral blood mononuclear cells (PBMCs) obtained from skin test-positive and -negative volunteers. The PBMCs from both donor groups produced elevated levels of inflammatory cytokines upon exposure to FKS, which could account for the discomfort generated by vaccine in humans [2]. BALB/c mice immunized subcutaneously with the FKS vaccine typically survive a lethal, intranasal challenge of *Coccidioides*, but fail to clear the pathogen from their lungs. A similar observation was made in BALB/c mice immunized with the formalin-killed Δ*chs5* strain (Fig. 2). Although 40% of the immunized mice survived beyond 45 days post-challenge, all of these animals showed persistent (chronic) infection in their lungs. Investigations of the profile of immune response of BALB/c mice immunized with the live Δ*chs5* vaccine and then challenged with *Coccidioides* by the intranasal route, will help to provide an understanding of the factors that contribute to protective immunity against coccidioidal infection. These studies are fundamental to the development of an effective coccidioidomycosis vaccine. However, the use of viable or non-viable, undefined, multicomponent immunogens as vaccines is problematic, not only because of potential toxicity issues, but because compositional changes in different preparations of the immunizing reagents could induce significant variations in host immunoreactivity. Current efforts to develop a vaccine against coccidioidomycosis, therefore, have focused on the identification of purified antigens which elicit a protective immune response.

Th1/Th2 pathways of immune response to coccidioidal infection

Both clinical and experimental evidence have demonstrated that T-cell immunity is pivotal for defense against coccidioidomycosis [96]. Two functionally distinct subsets of $CD4^+$ T cells have been identified and are distinguished by different patterns of secreted cytokines [97]; T helper 1 (Th1) lymphocytes are characterized by production of interleukin-2 (IL-2), IL-12, tumor necrosis factor (TNF)-α, and gamma interferon (IFN-γ), while T helper 2 (Th2) cells produce IL-4, IL-5, IL-6, and IL-10. Cytokines play a major role in the activation of the Th1 and Th2 pathways of immune response, and many of the T-cell receptors and signal transduction events associated with Th1/Th2 cell differentiation have been characterized [98]. These two cytokine profiles correlate with resistance and susceptibility to *Coccidioides* infection, respectively [96,99–101]. Th1 cells secrete cytokines that initiate and participate in cell-mediated immune responses, including the activation of macrophages, while the Th2 subset of T lymphocytes secrete cytokines that stimulate B-cells to produce antibodies, activate mast cells and eosinophils, and may down-regulate cellular immune responses [102]. Th2 immunodominance compromises host protection against coccidioidal infection and exacerbates the course of disease [103]. Huffnagle and Deepe [104] have pointed out that host response to

systemic fungal infections "is the outcome of an interplay between innate immunity, adaptive immunity (Th1, Th2, T regulatory cells, B cells and antibodies) and fungal virulence factors."

Alveolar macrophages are most likely among the first innate cells to encounter and engulf arthroconidia of *Coccidioides*, but in vitro studies have suggested that the majority of these fungal cells survive [21,105]. The ability of the arthroconidium to withstand the attack of macrophages has been suggested to be at least partly due to the impermeable nature of its outer conidial wall layer, which is rich in hydrophobins [106]. The latter are cysteine-rich proteins that typically occur as linear, crystalline structures (rodlets) at the surface of air-dispersed conidia produced by most filamentous fungi. A mutant strain of *Aspergillus fumigatus* which lacks rodlets has been shown to be more sensitive to killing by alveolar macrophages than the parental strain [107]. Arthroconidia of *Coccidioides* germinate in vivo by undergoing isotropic growth and differentiation into large, multinucleate spherules, approx. 40–100 μm in diameter. At this stage, the parasitic cells are too large to be engulfed by host phagocytes. Each spherule subsequently endosporulates to produce ≥ 200 endospores (see Fig. 1). Amongst the medically important fungi, this high reproductive capacity in vivo is a feature which is peculiar to *Coccidioides*, and undoubtedly contributes to its ability to infect and colonize the host.

The size of the inhaled arthroconidial inoculum and intrinsic factors that govern the immune status of the host [108] are pivotal in determining the outcome of disease. To exemplify this point, BALB/c and C57BL/6 mice were first immunized with a recombinant, proline-rich antigen (Ag2/Pra), one of the vaccine candidates currently under investigation [109]. The mice were then challenged intranasally with different amounts of arthroconidia. Protection in vaccinated BALB/c mice was lost when more than seven arthroconidia were used for intranasal inoculation, while vaccinated C57BL/6 mice were still protected when challenged with a 20-fold-larger number of spores [56]. A contributing factor to this difference in susceptibility is that naïve C57BL/6 mice develop a predominant Th1 response to infection, while BALB/c mice mount a major Th2 response. Dendritic cells (DCs) are key players in the host innate cell repertoire, and may influence this difference in immune response between the two inbred strains of mice. DCs are one of the earliest cell types to be exposed to the pathogen, are the most potent antigen-presenting cells (APCs), and have the ability to activate lymphocyte response against the microbial infection [110]. *Coccidioides* antigens have been shown to activate immature DCs in vitro to become mature DCs [111]. The latter are characterized by increased expression of major histocompatibility complex II (MHC class II) molecules at the cell surface, production of Th1-type cytokines, up-regulation of costimulatory molecules, and increased ability to activate T cells through Toll-like receptors (TLRs) [104]. DC and other host cell recognition of various microbial molecules is mediated by binding of pathogen-derived ligands to specific surface-expressed TLRs (currently 10 functionally distinct TLRs have been reported) [112]. Naïve splenic DCs from BALB/c mice have been shown to express elevated levels of TLR2, -4, -5, and -6 mRNA, while DCs from C57BL/6 mice preferentially express TLR9 [110]. When exposed to known TLR ligands from *Listeria monocytogenes*, DCs from BALB/c mice responded by production of large amounts of the chemokine, monocyte chemoattractant protein 1 (MCP-1), while DCs from C57BL/6 mice produced significantly higher levels of IL-12p40. MCP-1 has been reported to enhance Th2 differentiation in vitro and the Th2 pathway of immune response in vivo [113]. As indicated, the latter may contribute significantly to the susceptibility of BALB/c mice to disseminated coccidioidomycosis. Understanding the differences in basal expression of TLRs in BALB/c and C57BL/6 mice, as well as the specific roles of TLRs in innate antifungal response [114], may provide insights into the factors that contribute to differences in susceptibility to coccidioidomycosis within the human population.

The histopathology of disseminated coccidioidal infections in humans and mice shows similar features [103]. For example, both infected human and murine lung tissue reveal comparable interactions of host cells with *Coccidioides* spherules prior to and after endospore release [115] (Fig. 3A and B, respectively). At the pre-endosporulation stage, spherules are frequently found in association with damaged lung tissue (Fig. 3A). Recent evidence has been presented that damaged host cells release uric acid that forms crystals capable of activating dendritic cells [116]. The DCs, in turn, signal inflammatory cells and T lymphocytes to respond to the insult. This may be a second mechanism, in addition to microbial ligand-TLR interactions, for the host immune system to scan for signs of danger and strangers [117]. Prior to endospore release, the spherule outer wall (SOW) layer has been shown to be composed of a lipid/glycolipid/glycoprotein matrix [118–121]. This observation is in agreement with that of Target and Breslau [122], who first reported that the walls of spherules are rich in "lipid complexes." The potential significance of this SOW layer is that its lipid compo-

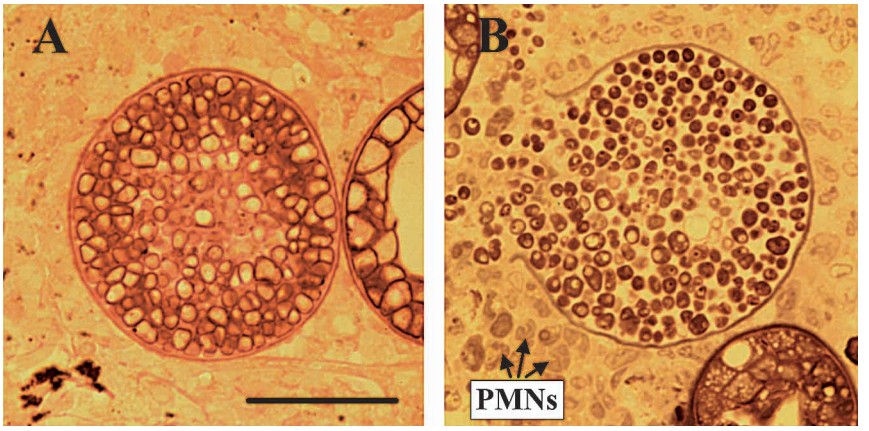

Fig. 3 Paraffin-sections of infected murine lung tissue, which show spherules of *C. posadasii* prior to and after endospore release (A and B, respectively). Note the intense host inflammatory cell response to the ruptured spherule (primarily polymorphonuclear neutrophils [PMNs]) (B). The bar in (A) represents 60 μm.

nents may be processed by specialized CD1-reactive T cells and presented as lipid antigens to lymphocytes with diverse T cell receptors (TCRs) [123,124]. It is now well established that CD1d molecules in mice present microbial-derived lipid antigens to T cells, suggesting that they participate in host defense against pathogens. The major population of CD1d-restricted T lymphocytes has been identified as $V\alpha14^{+}$ natural killer T cells, which apparently can function as APCs and have been shown to play a key role in the innate phase of host protection against microbial pathogens [125,126]. CD1d-reactive natural killer T (NKT) cells can produce Th1- and/or Th2-type cytokines and activate IL-12 production in response to initial microbial infection. NKT cells respond to early immune signals, augment APC IL-12 production, activate NK cells, and are implicated in the regulation of adaptive immune responses [127]. Mice which lack the CD1d receptor molecule ($CD1d^{-}/^{-}$) were evaluated for their susceptibility to the Lyme disease spirochete, *Borrelia burgdorferi*. The latter is an extracellular pathogen that expresses proinflammatory lipid antigens at its cell surface [128]. The CD1 knock-out mice exhibited impaired resistance to the spirochete pathogen. The possibility that CD1 may function as antigen-presenting molecules that bind and present lipid antigens of SOW during early stages of coccidioidal infection is worthy of exploration.

Isotropic growth of endospores within the maternal spherule results in pressure applied to the inner circumference of the spherule wall, which eventually causes it to rupture. As the contents of the endosporulating spherule are released, the host mounts an intense inflammatory response. Neutrophils are the dominant innate cells found at sites of endospore release (Fig. 3B) and most likely secrete both IL-12 and IL-10, a characteristic early response to fungal infections [129]. In fact, IL-12 is produced by a variety of APCs, including dendritic cells and macrophages. Co-administration of an IL-12 expressing plasmid to BALB/c mice, together with a cDNA vaccine encoding the Ag2/Pra antigen described above, has been shown to enhance the vaccine-induced protective immunity against experimental coccidioidomycosis by stimulation of Th1-associated immune responses [130]. IL-12 has a multiplicity of functions, which include the stimulation of T lymphocyte and natural killer cell proliferation, increased cytolytic activity of macrophages, and induction of secretion of Th1-type cytokines such as IL-2, TNF-α, and IFN-γ [99,131]. The relative amounts of IL-12 and IL-10 secreted in murine lung tissue during early stages of coccidioidal infection may significantly influence the outcome of disease. Although IL-10 production is important in regulating the synthesis and secretion of IL-12 by host innate cells [132], a persistent, elevated level of IL-10 may contribute to increased host susceptibility to disseminated coccidioidomycosis. *Coccidioides*-infected BALB/c mice produce more IL-10 and IL-4 during early stages of disease than inbred strains with higher resistance to infection (e.g., C57BL/6 and DBA/2) [133]. *Coccidioides*-infected, IL-10-deficient mice are as resistant to coccidioidomycosis as DBA/2 mice, suggesting that a persistent, high level of IL-10 production during coccidioidal infection has a net compromising effect on host immune response. The ability of IL-10 to suppress accessory cell functions required for optimal T-cell activation makes it a 'down-regulator' of cell-mediated immunity [134].

Little is known about the involvement of NK cells in host immune response to *Coccidioides* infections. Indirect evidence has suggested that NK cell components of human peripheral blood lymphocyte preparations inhibit growth of endospores in vitro, and that

this inhibitory effect can be enhanced by the addition of IFN-γ [135]. As indicated above in the discussion of the function of CD1d-restricted NKT cells, NK cells in general seem to play an important role in innate immunity against infectious diseases and in linkage to adaptive immunity [136]. Two subsets of human NK cells have been distinguished on the basis of their relative expression of CD56; CD56dim cells comprise the majority of NK cells that function as effectors of natural cytotoxicity and antibody-dependent cellular cytotoxicity, while CD56bright cells have immunomodulatory properties that function through secretion of cytokines, including IFN-γ [137]. Activated NK cells have also been shown to prime DCs to produce IL-12 [138], which is necessary for IFN-γ secretion by T lymphocytes and the elaboration of a Th1 response [104]. During the active phase of experimental *Histoplasma capsulatum* infection in C57BL/5 mice (<3 weeks post-challenge), macrophages and NK cell numbers sharply increased during the initial inflammatory response, followed by a rise in the $CD4^+$ T cell population [139]. The first cytokine transcripts to be detected in these infected mice were for IL-12 and IL-4, followed by IFN-γ. The authors showed that quantitatively, the increase in the IFN-γ transcript level was 2 logs higher than the transcript levels of IL-12 and IL-4 at about 3 weeks post-challenge. Similar observations have been made in resistant strains of mice infected with *Coccidioides* [96,99,101]. On days 9 to 10 post-infection with *H. capsulatum*, following the initial inflammatory response, the hierarchy of IFN-γ expression was $CD4^+ > CD8^+ >$ NK cells. These observations support the contention that cytokine secretion by NK cells plays an important supporting role in immunomodulation during early stages of infection.

Production of interleukin-4, -5, and -6 significantly influences the outcome of coccidioidomycosis. A persistent high level of IL-4 production in lungs of naïve BALB/c mice during stages of *Coccidioides* infection appears to down-regulate CMI. T helper cells are a major source of IL-4, although mast cells can also secrete this cytokine [113,132]. IL-4 knock-out mice have been shown to be more resistant to *A. fumigatus*, and shift from a Th2-type response to a Th1 response to infection [140,141]. Neutralization of endogenous IL-4 in *Coccidioides*-infected BALB/c mice by administration of neutralizing anti-IL-4 antibody led to a significant reduction of fungal burden in their lungs [101]. Persistently high levels of IL-5 and IL-6 have been observed in BALFs of non-immunized BALB/c and C57BL/6 mice between day 4 post-infection with *Coccidioides* and death of the animals (Fig. 4A, B). Eosinophilia is an indicator of coccidioidal infection,

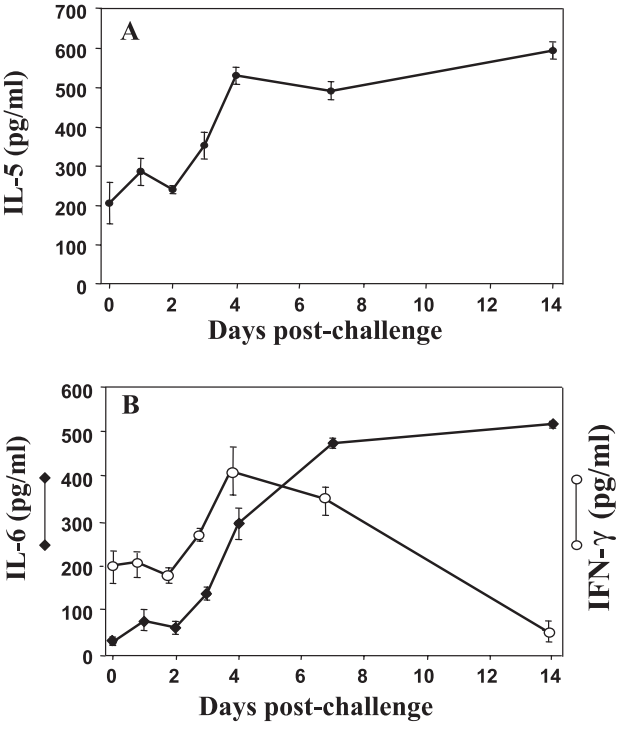

Fig. 4 Profiles of cytokine response of non-immunized C57BL/6 mice to intranasal challenge with *C. posadasii*, as determined by ELISAs of BALFs. (A) IL-5; (B) IL-6. Data were derived from 3 mice per time point. Mice were challenged with 80 arthroconidia. Similar profiles of cytokine response were observed in non-immunized, *Coccidioides*-infected BALB/c mice (not shown).

and is noted in approximately 25% of patients diagnosed with this fungal infection [103]. In fact, marked eosinophilia may be an important clue that dissemination of *Coccidioides* has occurred [142]. IL-5 is an eosinophil differentiation factor, and increased levels of IL-5 in bone marrow correlate with the appearance of increased eosinophil numbers in the blood of patients [143]. Extensive clinical evidence has suggested that eosinophils play an important role in the pathogenesis of asthma, which is characterized by Th2 immunodominance [144]. The key role of IL-5 in eosinophil production and function has made this cytokine a prime therapeutic target [145]. The use of IL-5 neutralizing antibodies in animal models of asthma has been shown to result in suppression of eosinophil recruitment to the lungs [146]. However, the effects of eosinophil recruitment to sites of inflammation may not be entirely negative. Eosinophils produce growth factors, and may play an important role in the resolution of inflammation and tissue repair. Thus, the effects of using antibodies raised against IL-5 to reduce eosinophilia, particularly in humans, requires careful study [145]. Nevertheless, IL-5 has been im-

plicated in the recruitment and activation of eosinophils within the lungs of the *Coccidioides*-infected host, resulting in localized tissue damage. Huffnagle and coworkers [147] have shown that development of chronic eosinophilia in the lungs of *Cryptococcus neoformans*-infected C57BL/6 mice is a non-protective immune response that causes significant lung pathology. In murine coccidioidomycosis, a persistently high level of IL-5 production is indicative of a dominant Th2 response to infection, and a poor prognosis of disease outcome. IL-6 is considered a pleiotropic cytokine [148]; it has been described as both a pro-inflammatory and anti-inflammatory molecule. Early IL-6 expression plays a pivotal role in the induction of host inflammatory response to infection. However, persistent antigen-driven, APC-derived IL-6 production can influence $CD4^+$ T cells to produce IL-4 and, thereby, promote Th2 differentiation and inhibit Th1 polarization. The latter has been suggested to occur by up-regulation of suppressor of cytokine signaling (*SOCS*) gene expression, which interferes with IFN-γ signaling and the development of Th1 cells [149]. IL-6 secreted by *Mycobacterium tuberculosis*-infected macrophages has been shown to inhibit the activation responses of uninfected macrophages to IFN-γ, which contributes to the inability of the cellular immune response to eradicate infection [150]. We have shown that an inverse relationship exists between the production of IL-6 and IFN-γ during progressive stages of coccidioidal infection in naïve mice, as measured by cytokine enzyme-linked immunosorbent assays (ELISAs) of BALFs over a 2-week period post-infection (Fig. 4B). This time course of reduction of IFN-γ expression in the lungs of infected mice correlates with dissemination of the pathogen.

Recent evidence has suggested that persistently elevated production of IL-6 may contribute to the inability of regulatory T cells to control the activation of T effector cells in response to the presence of the pathogen [151]. This, in turn, could lead to intense inflammation at sites of infection together with host tissue damage, and further exacerbation of disease. Interest in the function of regulatory T cells (T_R) in infectious disease is based on the current assumption that they play an essential role in prevention of harmful immune pathology during the course of microbial infection [152]. Most members of this small subset of $CD4^+$ T lymphocytes present in healthy individuals (approx. 5 to 10%) are distinguished by their expression of the activation marker CD25. However, it was the discovery that the transcription factor FOXp3 acts as the 'master control gene' for T_R cells, which defined this subset of lymphocytes as a distinct cell lineage [153]. These naturally occurring, thymus-derived T_R cells ($CD4^+CD25^+$) have the ability to inhibit the proliferation of T effector lymphocytes ($CD4^+CD25^-$) by their secretion of cytokines (e.g., IL-10), and remain functionally stable for long periods in the absence of antigen [154]. Typically, the roles of $CD4^+CD25^+$ T cells are to support the mobilization of T cell-mediated immune response against an invasive microbe, while simultaneously minimizing host tissue damage due to immune pathology. The activities of T regulatory cells during the course of infectious disease can also be a potential liability. For example, recent studies of murine infections with the malaria parasite *Plasmodium yoelii* have indicated that activation of T_R cells contributes to immune suppression and helps the parasite to escape from host immune responses [155]. Antibody depletion of $CD4^+CD25^+$ T_R cells protected mice from death when infected with a lethal strain of *P. yoelii.* During cutaneous infection of C57BL/6 mice with *Leishmania major,* $CD4^+CD25^+$ T cells have been shown to accumulate in the dermis where they suppress – by both IL-10-dependent and IL-10 independent mechanisms – the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite [156]. Depletion of $CD4^+CD25^+$ T cells led to clearance of the pathogen. However, the sterilizing immunity achieved under these conditions resulted in loss of immunity to reinfection, suggesting that an equilibrium is established between regulatory and effector T cells at sites of chronic *Leishmania* infection, which might reflect both parasite and host survival strategies. A similar scenario may exist during chronic infections with *Coccidioides*. A molecular signaling complex which appears to regulate the activities of $CD4^+CD25^+$ cells has been partially defined [154]. This discovery gives credence to the concept of 'contrasuppression' [157], which may be a particularly important factor in the development of protective immunity against *Coccidioides* infection.

Histopathological evidence suggests that host tissue damage occurs at sites of coccidioidal infection where the pathogen persists and high levels of IL-6 production are maintained. The concept that host response contributes significantly to pathogen-mediated damage of host tissue has been well articulated by Casadevall and Pirofski [158,159]. An intriguing proposal in this commentary is that regulation of immune response and generation of long-lasting protection against fungal infections may, paradoxically, require the production of both Th1 and Th2 cytokines to achieve a modicum of Th1/Th2 balance [160]. Opsonizing antibodies which bind to fungal pathogens have been suggested to significantly influence both the nature of innate cell-microbe interactions, as well as the development of

long-lasting antifungal immunity [161]. This concept is in opposition to current opinion of the importance of antibody in protection against coccidioidal infection. Antibody-mediated immunity (AMI) has always been thought to be of little benefit since high antibody titers in patients with coccidioidomycosis typically correlate with poor clinical outcome [78]. However, antibody and complement undoubtedly provide opsonic activity, and enhance ingestion of the pathogen by host phagocytes. Complement activation leads to release of molecular fragments that are chemotactic for PMNs [162] (see Fig. 3B), and opsonize arthroconidia and endospores for engulfment by phagocytic cells. Nevertheless, no evidence has been provided to date that a subset of antibodies to specific *Coccidioides* antigens is protective [103]. However, this conclusion is based primarily on clinical studies rather than extensive experimental evidence. Casadevall [163] has provided a thoughtful argument that the interactions of AMI and CMI influence the outcome of fungal infections, and should be viewed in the context of an integrated immune response [164].

Considerably more is known about cytokine regulation of Th1 and Th2 responses than the roles of chemokines in host response to fungal infections in general, and coccidioidomycosis in particular. Chemokines are small, mainly soluble cytokines that are chemotactic for leukocytes [165]. Since their discovery in the early 1980s, the number of chemokines has risen to almost 50 [166, http://cytokine.medic.kumamoto.u.ac.jp]. Members of the chemokine family fall mostly into two groups; the CC chemokines with two adjacent cysteines near the N-terminus, and CXC chemokines in which the equivalent two cysteine residues are separated by another amino acid [132]. These two groups of chemokines act on different sets of receptors. The CC chemokines bind to CC chemokine receptors (CCR1, CCR2, etc.), while CXC chemokines bind to CXC receptors (CXCR1, CXCR2, etc.). More than 20 chemokine receptors have been identified. Although it first appeared that the chemokine network incorporates a high degree of redundancy, results of murine gene-targeted chemokine and chemokine receptor knock-out studies have suggested that chemokine regulation is incredibly intricate [165,167]. Several chemokines/chemokine receptors have been the focus of attention in fungal infections because of their influence on chemotaxis of host innate immune cells, as well as Th1 vs Th2 polarization of immune response to the microbial insult. For example, Eotaxin-a is a chemokine that functions cooperatively with IL-5 in the recruitment of eosinophils to sites of inflammation [145]. During early stages of T cell activation, post-infection, naïve T cells in secondary lymphoid tissue express C-C chemokine receptor 7 (CCR7), which is a major chemotactic receptor for dendritic cells [168]. Activated T cells express CCR2, which may be involved in blood-to-lung migration of antigen-presenting cells, such as DCs and macrophages [169,170]. Polarized Th1 and Th2 cells are distinguished by their expression of high levels of CXCR3 or CXCR4, respectively [171]. CXCR3 is a major chemotactic receptor for activated Th1 T cells, while CXCR4 is a chemoattractant for Th2 cells. Traynor et al. [167] have reported that CCR2 is required for Th1 polarization in murine lungs infected with *Cryptococcus*. Based upon results of our early studies (unpublished), we suspect that expression of MIP-1α and CXCR3 (Th1-related), and MCP-1 and CXCR4 (Th2-related) play pivotal roles in trafficking of APCs and T cells during early stages of coccidioidal infection. Current studies of chemokine and chemokine receptor expression in mice at sites of lung infection with *Coccidioides* are ongoing in our laboratory. The techniques employed in these investigations are illustrated and discussed below under the section on measurements of host protection.

A summary of the major factors known to contribute to host immune defense against *Coccidioides* infection is illustrated in Fig. 5. The pathogen and host establish both an intracellular and extracellular relationship. Arthroconidia and endospores are phagocytosed, but the latter appear to be the most vulnerable to growth inhibition and killing by activated phagocytes. The response of $CD4^+$ T lymphocytes to infection has been assumed to be essential for protection against coccidioidomycosis, while the role of $CD8^+$ T cells in this process is unclear [103]. Although cytotoxic T lymphocyte (CTL) activity in coccidioidomycosis has not been reported, a role for $CD8^+$ T cells in host defense was suggested in the report that optimal protection by adoptive transfer of immune spleen cells to syngeneic mice occurred when both CD4 and CD8 cells were present [96]. Depletion of either subset reduced the level of protection conferred to the recipient. $CD8^+$ T cells are known to be activated in response to intracellular pathogens, and share some of the same effector mechanisms as $CD4^+$ T cells, including the secretion of IFN-γ [172]. It would seem logical, therefore, that $CD8^+$ T cells may play a role in cell mediated immunity under conditions of a reduced or absent $CD4^+$ T cell population (e.g., in patients with AIDS). Wüthrich et al. [173] demonstrated that mice depleted of $CD4^+$ T cells can be protected by vaccination against an extracellular or intracellular fungal pathogen, and the key players in this immune protection were the antigen-activated memory $CD8^+$ T cells. The latter

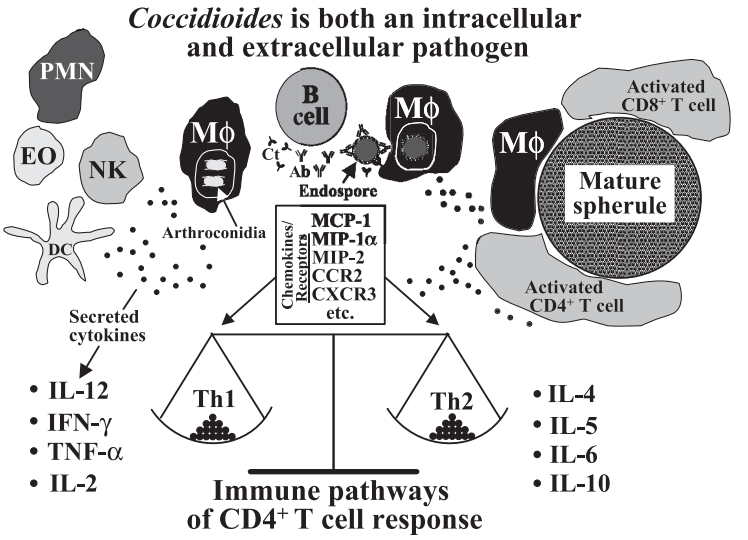

Fig. 5 Diagrammatic summary of murine innate and acquired immune response to *Coccidioides* infection. Ab, antibodies; CCR2, CC-type chemokine receptor 2 which interacts with MCP-1 (see below); Ct, complement component; CXCR3, CXC-type chemokine receptor which interacts with IP-10 (IFN-γ inducible protein); DC, dendritic cell; EO, eosinophil; IFN-γ, interferon-gamma; IL, interleukin; Mφ, macrophage; MCP-1, macrophage chemoattractant protein-1 (CC-type chemokine); MIP-1α, macrophage inflammatory protein-1 alpha (CC-type chemokine); NK, natural killer cell; PMN, polymorphonuclear neutropil; Th1/Th2, T helper 1/T helper 2 immune response pathways,; TNF-α, tumor necrosis factor-alpha.

were activated in a MHC I-restricted manner and secreted Th1-type cytokines to provide durable vaccine immunity to the mice. These results suggest that a therapeutic vaccine against systemic fungal infections could be used to immunize patients with $CD4^+$ T cell deficiencies. Identification of the key transcription factors which regulate development of functionally distinct T cell lineages is almost at hand [174], and the results of these studies will contribute significantly to our understanding of how $CD4^+/CD8^+$ T cell responses to microbial infections are coordinated. At this point, it is not known whether $CD8^+$ T cells can be stimulated by a coccidioidal vaccine to provide protection to an immunodeficient host, or whether a therapeutic vaccine against coccidioidomycosis in patients with reduced $CD4^+$ T cell numbers is feasible.

Adjuvants

Adjuvants include naturally occurring and synthetic, bioreactive products which are used for immune-potentiating activity that is optimally suited to vaccines developed against particular diseases. During the past few years, several authoritative reviews have been published on the discovery and delivery of vaccine adjuvants [e.g., [175–177]]. It is fair to state that "adjuvant design has historically had a touch of alchemy at its heart due to its reliance on the complex biology of innate immune activation" [177]. On the other hand, it is evident from the above discussion that substantial progress is being made toward a mechanistic understanding of innate immunity. With the discovery of the TLR family of receptors, many pathogen products with known immune-potentiating activity have been shown to activate innate immunity via TLR binding and/or through their interaction with an increasing number of non-TLR, innate cell-surface expressed pattern-recognition receptors (PRRs) [177]. Some TLRs seem to be highly promiscuous (e.g., TLR2 and TLR4), and have been shown to participate in response to numerous and diverse immune-potentiating reagents. In fact, TLR2 and 4 apparently play a more general role in the initiation and amplification of the early immune response, whereas other TLR family members have evolved more specialized functions [177]. For example, bacterial lipopolysaccharide (LPS) binds to the glycosylphosphatidylinositol (GPI)-anchored membrane protein CD14, and activates both TLR2 and TLR4 to induce an immune response [178,179]. Thus, CD14 functions as a PRR under these conditions, but lacks an intracellular signaling domain and requires the TLRs for transmission of the activating signal into the cell [180]. Overexpression of a constitutively active form of TLR4 in transgenic mice results in a significant increase in innate cell sensitivity to LPS both in vitro and in vivo [179]. Upon infection with *Salmonella typhimurium*, these transgenic mice produced markedly elevated levels of Th2 cytokines. The long-term adjuvant effect of LPS exposure revealed in these studies was the development of an excessive inflammatory response which was detrimental to the host. On the other hand, a detoxified form of bacterial LPS isolated from *Salmonella minnesota* has been shown to be an effective vaccine adjuvant. The

compound, known as monophorsphoryl lipid A (MPL®; mol. weight approx. 1718 daltons), has been shown to have greater Th1 than Th2 stimulating potential. MPL has been extensively evaluated as an adjuvant in human vaccine trials for malaria, hepatitis B, and allergy desensitization. It has been shown to be well tolerated, and enhances the efficacy of the vaccination protocol [181]. The benefits afforded by MPL in vaccine formulations are attributed to its immunostimulatory effects on the innate immune system, presumably mediated through the activation of both TLR2 and TLR4. MPL activation of APCs results in enhanced phagocytosis and microbicidal activities. The latter are the consequence of elevated levels of production of Th1-type cytokines, including IL-12, TNF-α and IFN-γ [182].

MPL has been used as an adjuvant together with recombinant Ag2/Pra to test the effectiveness of this experimental vaccine against coccidioidomycosis [109]. MPL was substituted for complete and incomplete Freund's adjuvants, which are not approved for human use, and alum (aluminum hydroxide) which elicits a dominant Th2 pathway of immune response [183]. BALB/c mice immunized with rAg2/Pra + MPL showed significantly better protection against *Coccidioides* infection, and higher levels of IFN-γ production than mice immunized with the recombinant protein alone [109]. As an alternative to MPL, IL-12 has been tested in combination with Ag2/Pra for its ability to mount a protective Th1 response against murine coccidioidomycosis [184]. Interleukin-12 is a heterodimeric cytokine composed of a 40-kDa and 35-kDa chain [184]. The antigen and cytokine (both chains) were delivered to BALB/c mice intramuscularly as cDNA constructs in a mammalian plasmid vector, and resulted in enhanced protection against *Coccidioides* compared to mice immunized with Ag2/Pra cDNA alone. The protective response was characterized by increased IFN-γ production and a high titer of Th1-associated, anti-Ag2/Pra-specific IgG2a antibodies. However, an approximately equal titer of Th2-associated, antigen-specific IgG1 antibody was also detected in mice that received both antigen and adjuvant, and immunization with the combined antigen plus adjuvant resulted in little reduction of the fungal burden in lungs of mice challenged by the intranasal (pulmonary) route compared to control animals. The authors suggested that intranasal delivery of the combined Ag2/Pra-IL-12 cDNA vaccine directly to the lungs may improve its effectiveness. The results of these studies have not yet been reported. A variation of this vaccine formulation still untested against coccidioidomycosis is the inclusion of the recombinant antigen and IL-12 protein admixed with alum for immunization by the conventional subcutaneous route. Previous studies have demonstrated that coadsorption of antigen and IL-12 to alum promotes both Th1-type cytokine production and high levels of the correlated (IgG2a) antibody response [185]. As indicated in the earlier discussion of Th1/Th2 polarization of immune responses to coccidioidomycosis, it is possible that both cellular and humoral immunity are required for potent and durable protection against coccidioidal infection. The clinical application of IL-12 as a vaccine adjuvant, however, has encountered several problems. The heterodimeric molecule is expensive, difficult to manufacture, and its safety in human clinical trials is still questionable [182].

Multiple recent studies have reported that synthetic oligodeoxynucleotides (ODNs) with CpG motifs provide enhanced immune response to co-delivered antigens [92,186–188]. Vaccine formulations that include CpG oligodeoxynucleotides combined with recombinant antigens of *C. posadasii* [189,190] and *A. fumigatus* [186] have proved to promote Th1 response and provide good protection against the respective mycoses. A recent report, relevant to our use of the live, avirulent vaccine of *Coccidioides* described above, showed that CpG ODN improved the safety, potency and durability of a live vaccine against cutaneous leishmaniasis [92]. CpG DNA binds to TLR9 [191] and is suggested to directly activate dendritic cells and macrophages and enhance production of cytokines that create a Th1-like milieu in lymphoid tissue [192]. The Th1 response in the presence of CpG includes elevated levels of IL-12, IFN-γ, TNF-α expression and production of antimicrobial nitric oxide [92,193]. Co-administration of CpG ODN with soluble protein antigens in incomplete Freund's adjuvant has been shown to promote Th1 responses in vitro [194] and in vivo against murine coccidioidomycosis [189,190]. The sequence of the immunostimulatory CpG ODN (5' to 3') used to immunize mice was TCCATGACGTTCCTGACGTT (CpG motifs are underlined). The synthesized products contain a phosphorothioate backbone to ensure DNA stability in vivo. CpG ODN has proven to be superior to other well-tested adjuvants for its ability to induce Th1-biased immune responses. A major advantage of this DNA adjuvant is its apparent lack of toxicity in primates, although repeated administration (7–20 days) of high amounts of CpG ODN to mice (60 μg/dose) resulted in immunotoxicity and hepatoxicity [195]. Nevertheless, results of early clinical studies of CpG DNA have indicated that CpG ODN is tolerated by humans, works well in the immature immune system

of neonates, and is a strong mucosal adjuvant when delivered intranasally [196].

Measurements of host protection

As previously pointed out, protection of coccidioidomycosis-susceptible BALB/c mice against a lethal inoculum delivered via the intranasal (i.n.) route is the most stringent evaluation of the effectiveness of a candidate vaccine. For preliminary screens of the efficacy of a vaccine protocol, we routinely challenge immunized mice by the intraperitoneal (i.p.) route, and then sacrifice the animals 12–14 days post-inoculation to determine the fungal burden in their spleen and lungs [189,190,197]. The pathogen apparently migrates to the lungs from the site of i.p. inoculation, and marked reduction in colony-forming units in this organ has proved to be a good indicator of vaccine efficacy. The i.p challenge mode provides more precise control of the size of the inoculum actually delivered to the host, and achieves better reproducibility of levels of coccidioidal infection in the lungs than by the intranasal route of inoculation. The results of a representative study of fungal burden in the lungs and spleen of immunized and non-immunized mice challenged via the i.p. route versus the i.n. route are shown in Fig. 6. The numbers of colony-forming units (CFUs) per lung are expressed on a log scale. Because these values do not fall into a normal distribution, the Mann-Whitney *U* test was used to compare the median counts. Statistical significance of survival differences between groups of mice (at least 10 animals/group) were calculated by the Kaplan-Meier method.

An unexpected observation in this evaluation of the particular vaccine candidate was that the 1 μg dose of the recombinant protein plus adjuvant (i.e., 1 μg rGel1+CpG/IFA) elicited a more protective immune response than the 5 μg dose [190]. This observation was consistent in both the fungal burden and survival studies (Fig. 6A–C). Although the difference in survival using these two doses was not statistically significant, the consistency of results in animals immunized with 1 μg of rGel1 suggested that this vaccination dose indeed provided superior protection than the higher dose of recombinant protein. We further explored this difference in immune response by examination of cytokine production in these two groups of immunized and challenged mice compared to the non-immune, infected controls. The results suggested that immunization with 1μg of rGel1 stimulated an early Th1 pathway of immune response, indicated by higher levels of IL-12 (Fig. 6D) and IFN-γ production, and an increased level

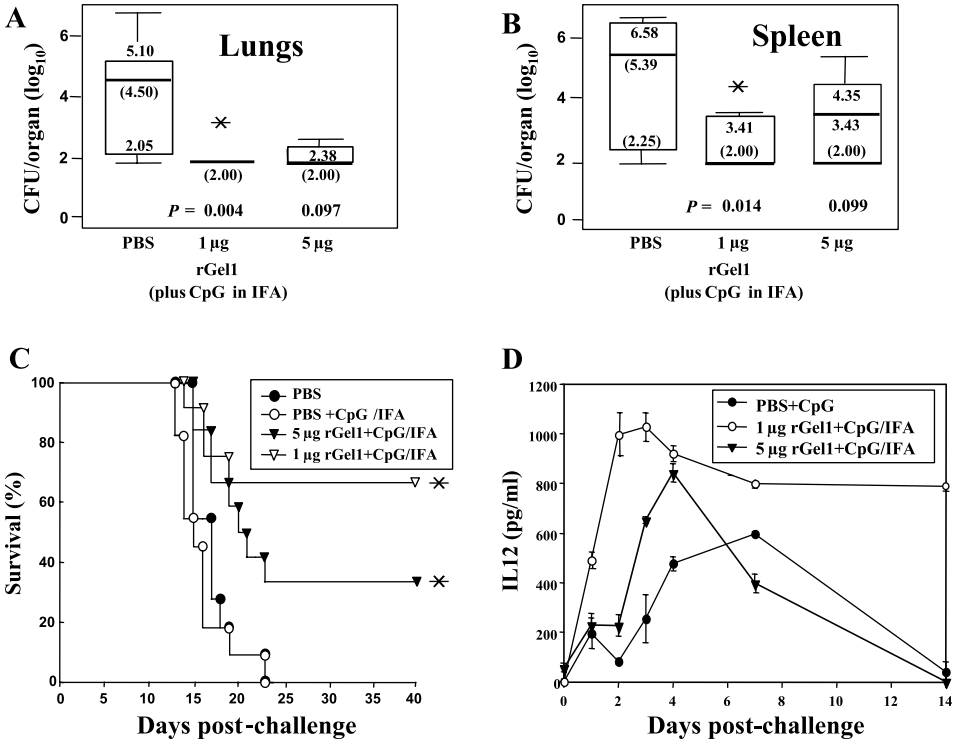

Fig. 6 Evaluations of fungal burden in lungs (A) and spleen (B) of BALB/c mice, and survival (C) of C57BL/6 mice immunized with recombinant Gel1 (either 1 μg or 5 μg per dose) plus adjuvant (CpG+IFA) and challenged with a lethal inoculum via either the intraperitoneal route (A,B; 100 arthroconidia) or the intranasal route (C; 80 arthroconidia). Groups of 12 mice were used for these two studies. Asterisks indicate a significant difference between rGel1-immunized and non-immunized mice (adjuvant or PBS alone). (Fig. 6A–C reproduced with permission from the American Society for Microbiology; taken from reference 190, Infect. Immun). (D). Profiles of IL-12 production by rGel1-immunized C57BL/6 mice (1 μg dose vs. 5 μg dose) compared to non-immunized mice, as determined by ELISAs of BALFs (A–C). Three mice per time point were assayed for each immunization protocol.

of rGel1-specific IgG2a titer when compared to infected mice immunized with the 5 μg dose. In fact, the latter group of mice appeared to respond to infection in a Th2 manner, with elevated levels of IL-5 and IL-10 production and high titers of rGel1-specific IgG1 antibody. In the absence of data on the distribution of Th1 and Th2 epitopes in rGel1, it is premature to speculate about the reason for this difference in immune T cell reactivity. However, the results of this experiment illustrate the need to examine the profile of cytokine expression as well as other indicators of a protective vs. non-protective response in immunized mice at different times post-challenge in order to critically evaluate the potency and durability of the candidate vaccine.

We have used two methods to examine the cytokine profile and thereby evaluate the protective efficacy of a candidate vaccine at selected times post-challenge: by quantitative analysis of cytokine proteins in bronchoalveolar lavage fluid of immunized, infected mice, and by quantitative analysis of cytokine gene expression in isolated, cryosectioned lung tissue. As discussed above, immunization with 1-μg vs. 5-μg doses of rGel1 induced different cytokine profiles in C57BL/6 mice post-challenge. In Fig. 6D, we compared the amounts of IL-12 detected in BALFs (as determined by ELISAs) over a two-week period post-challenge in vaccinated versus control mice. The sharp reduction in the amount of IL-12 present in mice immunized with 5 μg of rGel1 between days 4 and 14 post-challenge contrasted with the relatively high levels of this cytokine in mice immunized with 1μg of rGel1. These results correlated with differences in fungal burden (Fig. 6A,B) and survival (Fig. 6C). A semi-quantitative, but less time-consuming and more cost-effective method to initially evaluate changes in cytokine profiles can be conducted using commercially-available monoclonal/polyclonal antibody arrays to capture selected proteins from the BALF preparations [198,199]. The advantages of using these protein arrays are that many cytokines can be detected simultaneously using a membrane format, and the sensitivity and detection range are comparable to ELISA. We have found that the murine cytokine arrays are valuable adjuncts to cytokine profile analyses, and permit initial identification of individual cytokines in BALF preparations which can then be more precisely and critically examined by ELISAs.

The detection of cytokines in BALFs provides an indication of the nature of the total immune response in the lungs of i.n.-challenged mice, which for a particular murine strain will vary depending on the amount of original inoculum that reached the lungs and the degree of the infection within the lobes of the lungs. As an alternative to these assays, we have used laser capture microdissection (LCM) combined with quantitative, real time-PCR (QRT-PCR) to evaluate the local, in situ amounts of specific cytokine mRNA in host cells located adjacent to sites of coccidioidal infection in freshly prepared cryosections of murine lung tissue [200–202]. It is reasonable to assume that examinations of cytokine expression levels in innate cells and activated T lymphocytes adjacent to the pathogen in tissue sections provide accurate evaluations of the nature of localized host response to infection. The LCM procedure in our laboratory is conducted using an Arcturus PixCell IIe microscope equipped with an infrared diode laser, as well as fluorescence and bright-field illumination (Arcturus, Mountain View, CA). Immunized or non-immune control mice infected with *Coccidioides* are injected intravenously with 50 μl of a fluorochrome (Blankophor, 1 mg/ml) 30 min prior to sacrifice. The stain has been shown to reach the lungs and bind specifically to the fungal cell wall [203]. Blankophor is nontoxic and has no recognized effects on host immunity. Frozen sections of infected lung tissue (approx. 8 μm thick) are obtained from vaccinated mice, fixed, and used as the source of total RNA for subsequent studies. The advantage of in vivo staining of *Coccidioides* cells is that it permits identification of infection sites in lung sections by fluorescence microscopy, and minimizes the washing steps and loss of RNA prior to the microdissection and capture procedures. The laser beam is used for separate dissections of either the pathogen or the adjacent host tissue (Fig. 7). The resolution of fluorescence microscopy is adequate to distinguish between different stages of the parasitic cycle (see Fig. 1). The laser-dissected spherules or host tissues are captured by contact with the surface of a patented film attached to the cap of an Eppendorf microcentrifuge tube containing the reagents for RNA extraction. The total RNA is reverse-transcribed and used as the template for QRT-PCR assays of expression of either selected *Coccidioides* genes or host cytokine genes (Fig. 8). LCM-derived RNA has also been used to profile gene expression by employing high density oligonucleotide arrays [204]. This is a powerful method which can be used to generate an unbiased survey of the host transcriptome at sites of infection in lung tissue.

As previously indicated, ELISA determination of the relative titers of vaccine antigen-specific IgG2a vs. IgG1 is also a valid predictor of a protective or non-protective immune response against coccidioidal infection [109,189,190]. Comparative histopathology is likewise a valuable assay of the effectiveness of candidate vaccines in stimulating the host to sequester and clear

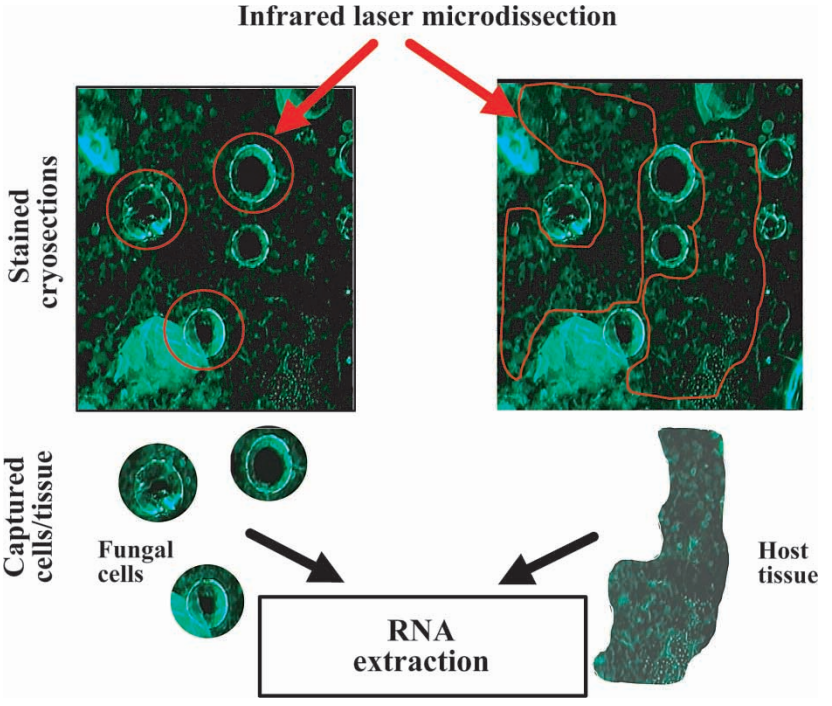

Fig. 7 Diagrammatic representation of methodology used to obtain RNA from either *C. posadasii* or host tissue isolated by laser capture microdissection (LCM) from cryosections of infected murine lungs.

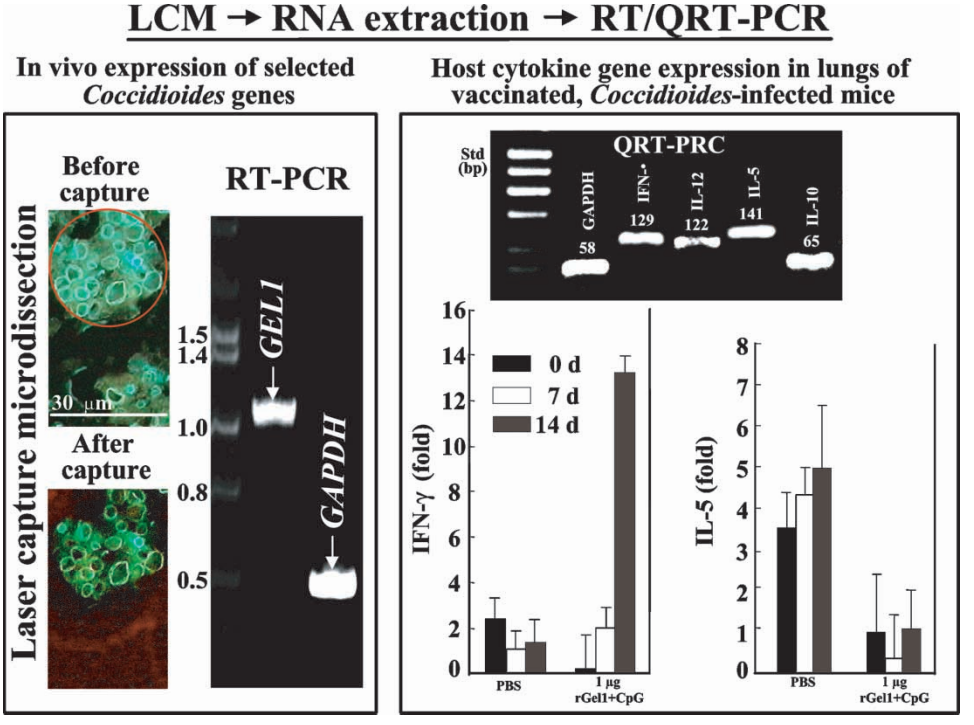

Fig. 8 Examples of semi-quantitative analysis of *C. posadasii* gene expression and host cytokine gene expression by reverse transcription-PCR and quantitative, real time-PCR (QRT-PCR). Template RNA was obtained by LCM from sites of infection in murine lungs.

the pathogen. The detection and characterization of granulomas in infected lungs of mice (determination of granuloma numbers, size and organization, presence/absence of giant cells, and relative amount of collagen using a collagen-specific dye) [205] may help to further provide a meaningful evaluation of vaccine efficacy.

Vaccine candidates

As previously stated, current efforts to develop a vaccine against coccidioidomycosis have focused on the identification of purified antigens which elicit a protective immune response. The majority of protective antigens that have been characterized to date are products of the parasitic phase of the fungus [103]. The most promising of these include two defined, cell wall-associated antigens [56,190]. Early studies have revealed that immunoprotective components of the spherule wall could be isolated by alkali treatment [206], or toluene extraction followed by deglycosylation with hydrogen fluoride [207]. The Cox laboratory cloned a protein that was present in the alkali-soluble, water-soluble preparation of the spherule wall, and referred to it as antigen 2 (Ag2) [208,209]. This nomenclature was based on a reference system which relies on two-dimensional immunoelectrophoretic separation of crude preparations of saprobic and parasitic phase antigens designated coccidioidin and spherulin [210]. The *AG2* gene was reported to encode a proline-rich protein with a predicted molecular mass of 19.4 kDa. The deduced protein sequence contains 10 repeats (TXX'P), predicted signal peptide and C-terminal GPI anchor, 8 cysteine residues, and 24 potential *O*-glycosylation sites. Simultaneous investigations of vaccine candidates in the Galgiani laboratory identified a protective 33-kDa proline-rich antigen (Pra) in SDS-PAGE gel separations of a hydrogen fluoride-deglycosylated lysate of *Coccidioides* [211]. The *PRA* gene was cloned and shown to be identical to the *AG2* gene sequence. The difference in molecular size of the predicted and electrophoretically separated, deglycosylated native protein is accounted for by its high proline content. Proteins with a high percentage of proline typically show anomalous molecular sizes when estimated on the basis of SDS-PAGE separations [212]. The recombinant antigen (rAg2/Pra) was expressed by *E. coli* and shown to elicit both T cell and antibody responses in patients with coccidioidomycosis [213–215]. Immunization of BALB/c mice with rAg2/Pra plus Freund's adjuvant protected the animals against i.p. challenge with 50 arthroconidia of *C. posadasii*, based on reduction of the fungal burden in the lungs and spleen at 14 days post-infection [216]. CFUs in the immunized mice were reduced by 2–2.5 $\log_{10}$ units compared to the controls. Northern blot analysis of *AG2/PRA* gene expression revealed that levels of specific mRNA increased during stages of spherule maturation [217], which correlated with the immunolocalization of the antigen in the parasitic cell wall [218]. In addition, little allelic diversity was detected in the gene isolated from multiple strains of *C. posadasii*, suggesting that the rAg2/Pra vaccine should provide comparable protection against a wide range of isolates. This same antigen was also expressed in bacteria as a glutathione-S-transferase (GST) fusion protein and demonstrated protection against i.p. challenge in BALB/c mice as assessed by decreased fungal burden [130]. However, in a survival experiment, BALB/c mice immunized with this recombinant antigen failed to show protection compared to control mice during a 30-day period post-challenge [130].

These contradictory results of the protective efficacy of the recombinant Ag2/Pra in BALB/c mice have raised doubts about the durability of protection afforded by this immunogen. Recent studies have suggested that the protective epitopes of Ag2/Pra are contained within the N-terminal region of the protein (amino acids 1–106), including the putative, 18-residue signal peptide [219,220]. BALB/c mice immunized with a plasmid vector-expressed N-terminal fragment of Ag2/Pra and challenged via the intraperitoneal route showed reduced fungal burden in their lungs and spleen compared to non-immunized control animals 130, 220. On the other hand, BALB/c mice which were administered the same genetic vaccine and then challenged by the pulmonary route showed no significant reduction of fungal burden in their lungs, and their survival rate was the same as control animals [130,220]. It appears that both the recombinant protein and DNA formulations of the Ag2/Pra vaccine provide early protection to BALB/c mice against coccidioidomycosis, but fail to sustain protection against pulmonary infection when the animals are challenged with approximately 10–100 viable arthroconidia via the natural, i.n. route [56,130,220]. The residual pulmonary infection in these mice may provide a nidus for progressive disease and death of the animals, or the development of persistent pulmonary coccidioidomycosis commonly found in humans [221]. A potential problem related to chronicity of coccidioidal infection is that a shift from a protective Th1 to a non-protective Th2 immunodominance may occur, which could result in disseminated disease. It is also possible that this shift in immunodominance could be influenced by the acquired immunity induced during the prophylactic vaccination regimen. The protective N-terminal fragment of rAg2/Pra contains both Th1

and Th2 epitopes. As in the case of the tuberculosis BCG vaccine [222], the vaccination dose of rAg2/Pra appears to define the Th1/Th2 nature of the immune response. Immunization with low doses of rAg2/Pra (0.5–1μg) may elicit primarily a Th1 response, while higher doses (e.g., 5 μg) could induce mixed Th1/Th2 responses. These observations raise important questions about the durability of the rAg2/Pra vaccine that still need to be addressed.

A second promising protective recombinant protein is a wall-associated, GPI-anchored β-1, 3-glucanosyltransferase (rGel1) [190]. Antiserum raised against the purified protein revealed that the antigen is expressed on the surface of endospores, a stage of the parasitic cycle which can be engulfed by phagocytes. The recombinant protein is recognized in immunoblots by sera from patients with confirmed *Coccidioides* infection, but not with human control sera. These data indicate that Gel1 is a clinically relevant antigen. Immunization with rGel1 plus CpG/IFA elicited a protective response against coccidioidal infection via the i.p. and i.n. routes in both BALB/c and C57BL/6 mice. Immune mice challenged intraperitoneally with the pathogen showed approximately 3–4 $\log_{10}$ units reduction in fungal burden in their lungs and spleen, and when challenged by the i.n. route, 70% of the immune mice survived compared to none of the non-immunized animals.

As in the case of the recombinant Ag2/Pra vaccine, we have shown that immunization of mice with different amounts of rGel1 plus adjuvant results in different degrees of protection (Fig. 6A–C). In contrast to rAg2/Pra, however, we have revealed that vaccination of mice with the optimal amount of rGel1 (1 μg/dose) resulted in the maintenance of elevated expression levels of IL-12 and IFN-γ in BALFs and lung tissue of infected mice (Fig. 6D, Fig. 8), which is indicative of a sustained Th1 response.

A third recombinant protein that shows promise as a vaccine candidate is the *Coccidioides*-specific antigen (Csa) which we had isolated several years ago [48], but only recently tested for its ability to protect mice against coccidioidal infection [223]. The recombinant Csa protein was expressed by *E. coli* and used as a vaccine in combination with rAg2/Pra expressed by *Saccharomyces cerevisiae*. C57BL/6 mice were immunized intradermally with the combined vaccine plus CpG/MPL adjuvant. Survival experiments showed that rCsa + rAg2/Pra-immunized mice challenged intranasally with a lethal inoculum of *Coccidioides* showed 90% survival at 60 days post-infection compared to none of the infected animals immunized with adjuvant alone ($P < 0.0003$). Mice immunized with rCsa or rAg2/Pra alone plus adjuvant showed 30% and 60% survival, respectively. Multivalent recombinant vaccines may enhance protective immunity against coccidioidomycosis. It can be argued that such a vaccine may increase the probability of induction of an appropriate immune response in humans that leads to sequestration and clearance of the pathogen [224].

Most of the other recombinant antigens of *Coccidioides* which have been produced and tested to date have failed to meet the benchmarks of protection established in murine vaccine trials using rAgs/Pra and rGel1, and are no longer considered candidates for a human vaccine. These include the first purified recombinant T-cell-reactive protein of *Coccidioides* tested in a murine model [225], a heat shock protein (rHsp60] [226], and a spherule outer wall glycoprotein (rSOWgp) [103,121].

DNA vaccines against coccidioidomycosis which have been tested to date provide consistently better protection than recombinant protein vaccines [109,130,220]. This was well illustrated in mice immunized with recombinant urease (rUre) protein vs. the *URE* DNA vaccine [189]. The maximum survival observed in BALB/c mice immunized with the recombinant protein + CpG/IFA was 40%, while 80% of the animals survived after immunization with the pSecTag2A.*URE* plasmid construct. The apparent strong, polarized Th1-type $CD4^+$ and $CD8^+$ responses induced in mice by this DNA vaccine, coupled with its adjuvant activity, provide a cogent argument that DNA vaccination is a promising new approach for immunoprophylaxis against coccidioidomycosis. DNA vaccines are relatively easy to produce and less expensive than recombinant protein vaccines, do not require sophisticated purification schemes or a cold chain for storage, and can be used for repeated boosting [227]. However, evidence from clinical trials suggests that DNA vaccines have failed to induce the same level of robust and broad-based immune responses in primates, including humans, that have been reported in mice [227,228]. To date, no DNA vaccines have been approved for general human application in the U.S.

The Valley Fever Vaccine Project – a cooperative research effort

A consortium of researchers affiliated with five academic institutions in the United States have focused their efforts on development of a prophylactic vaccine against coccidioidomycosis. Much of the progress made over the last five years towards this goal has been the result of the integrated research activities of the consortium. An exciting approach to new vaccine

discovery which involves cDNA expression library immunization (ELI) is underway in the Cox laboratory (University of Texas Health Sciences Center, San Antonio) [229]. A *Coccidioides* spherule-phase cDNA library containing 800–1000 genes was divided into 10 pools, and each was tested for its protective efficacy in BALB/c mice against i.p. challenge with a lethal inoculum of the pathogen. Sequential fractionation of protective pools yielded a single clone, designated ELI-Ag1. The gene has been sequenced and expressed, and is under investigation as a vaccine candidate.

Our research group has cooperated with the Kirkland laboratory (Veterans Medical Research Foundation, San Diego) and Gardner laboratory (The Institute for Genomic Research [TIGR]) on use of the partial genome database of *Coccidioides* for the identification of genes that encode homologs of reported wall-associated proteins [103] and parasitic phase-specific antigens [230]. Our rationale for these discovery efforts is that some of the gene products may be immunogenic and exposed to the host immune system during the parasitic cycle. As discussed above, studies of candidate vaccines against coccidioidomycosis have indicated that the most protective antigens are parasitic cell wall proteins. We used the computer program BLASTx [231] to perform conceptual translations of nucleotide sequences in the *C. posadasii* genomic database, followed by searches of the nonredundant protein database [232] to find good alignments between the *Coccidioides* and database sequences [190]. Glimmer is a computational gene finder that has been helpful for *Coccidioides* gene identification and genome annotation [233]. Genomic studies have already contributed to the discovery of new arrays of genes involved in cell wall formation in *Saccharomyces* [234], expression of phase-specific antigens in *H. capsulatum* [235], and virulence factors in other microbial pathogens [236]. The analysis of single genomes is no longer satisfactory; comparisons of multiple genomes of pathogenic fungi (e.g., *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *H. capsulatum*, *Blastomyces dermatitidis*) provide insights into conserved or unique families of proteins and functional domains, improve the precision of annotation, and permit identification of novel immunogens which can be tested as vaccine candidates [237].

Crude subcellular vaccines against coccidioidomycosis have been reported [206,238]. Although these have promise because of their protective nature (e.g., Fig. 9A), they are unlikely to transition to clinical trial because the immunogenic components are undefined. However, proteomic methods are now at hand which can potentially overcome this problem [239]. Two dimensional-polyacrylamide gel electrophoresis (2D-

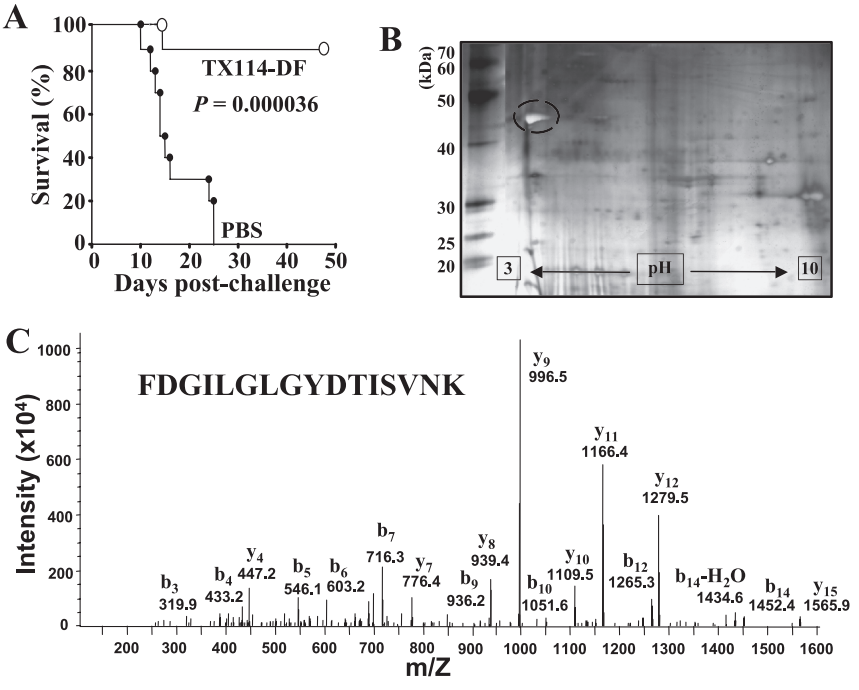

Fig. 9 Application of proteomics to vaccine discovery. (A). Survival plot of BALB/c mice immunized subcutaneously with a Triton X-114 detergent extract of an endosporulating spherule homogenate (TX114-DF; 14 μg x2) plus adjuvant (CpG+IFA) and challenged by the intranasal route with 90 arthroconidia (*C. posadasii*; strain C735). Control mice were immunized with adjuvant alone. The difference in percent survival between these two groups is significant. (B). 2D-PAGE separation of the TX114-DF immunogen, showing a major protein (circled) which was isolated and subjected to trypsin digestion. (C). Representative collision-induced dissociation (CID) spectrum generated by tandem mass spectrometric analysis of the peptides released from the digested protein in (B). The peptide sequence in (C) matches the reported sequence of a *Coccidioides* aspartyl protease (GenBank accession number AF162132).

PAGE) is used first to separate the total protein fraction (Fig. 9B). Isolated proteins excised as gel plugs are then subjected to in-gel trypsin digestion. Tandem mass spectrometry (MS) is subsequently employed to obtain amino acid sequences of the eluted peptides. The sequence data obtained from the resulting collision-induced dissociation (CID) spectra (Fig. 9C) can be used to search for sequence matches in the translated *Coccidioides* protein database. This approach to the systematic analyses of potential immunogenic components of a TritonX-114 detergent extract of the spherule wall fraction has resulted in the identification of a 45-kDa aspartyl protease (Asp1). This same protein was previously isolated in the Pappagianis laboratory (University of California at Davis) from a soluble vaccine prepared from formalin-killed spherules of *Coccidioides* [240]. The deduced protein sequence of the *ASP1* gene revealed 83.2% similarity to the amino acid sequence of a wall-associated aspartyl protease of *A. fumigatus* [241]. Homologs of the *C. posadasii* Asp1 have also been reported in *C. neoformans* (F. Dromer, Pasteur Institute, per comm.) (77.8% sequence similarity), and *Shistosoma japonicum* [242,243] (66.9% sequence similarity). In both cases, the aspartyl protease homologs have been shown to be T-cell reactive and suggested to be candidate vaccines against the respective microbial infection. Preliminary studies in our laboratory have shown that rAsp1 of *C. posadasii* protects C57BL/6 mice against a lethal i.n. challenge of the fungus. Compositional and structural studies of the proteome of *Coccidioides* represent the next frontier of research on this pathogen, and could lead to the discovery of the optimal vaccine which provides potent and durable protection against coccidioidomycosis.

Summary

Coccidioides is a formidable human pathogen which is capable of establishing a life-threatening respiratory disease in immunocompetent, healthy individuals. Naïve, adult BALB/c mice are highly susceptible to coccidioidomycosis when challenged by the intranasal route (<10 arthroconidia establish disseminated disease and can be lethal). BALB/c mice immunized subcutaneously with a live, avirulent mutant strain of *C. posadasii*, which was generated by disruption of a single chitin synthase gene, were protected against an intranasal challenge with 100 viable arthroconidia of the highly virulent, parental strain (C735). The phenotype of the Δ*chs5* mutant was characterized by aberrant hyphae both in vitro and in vivo. The results of this protection experiment demonstrate that successful vaccination against coccidioidal infection of a susceptible host challenged via the natural route with a lethal inoculum is possible, and that protective antigens are expressed by the saprobic phase and shared with the parasitic phase in vivo. This immunized mouse provides a model for examination of the host protective response to *Coccidioides* infection. The murine model permits studies of the phenotype of host cells involved in sequestration and clearance of the pathogen, and investigations of the expression of signal molecules (cytokines, chemokines, chemokine receptors) involved in the chemotaxis and activation of these cells during the innate and acquired immune response. We suggest that the results of these experiments will contribute significantly to the development of a human vaccine against coccidioidomycosis.

Although it is possible that a live, avirulent strain of *Coccidioides* could be developed as a human vaccine, it is more likely that a well-defined, recombinant protein-based vaccine would be approved by regulatory boards in the U.S. and accepted by volunteers in clinical trials. Therefore, the focus of our studies of candidate vaccines has been the identification of purified antigens which elicit potent and durable host protective response against *Coccidioides* infection. The lead vaccine candidates include rAg2/Pra, rGel1, and rCsa+rAg2/Pra. Potential candidates are in early stages of evaluation [e.g., recombinant protein expressed by ELI-Ag1 [229], and rAsp1]. Controversial issues related to the durability of protection afforded by vaccination with rAg2/Pra, especially in BALB/c mice, raise concerns about the use of this antigen in a human vaccine trial. Both rAg2/Pra and rGel1 require further evaluation in murine models to determine whether vaccine protocols using these separate recombinant proteins plus CpG/MPL can induce long-lasting protection and clearance of the pathogen. Early results of the protective efficacy of the combined rCsa+rAg2/Pra vaccine are very promising, and suggest that combinations of other recombinant proteins currently at hand may be equally or even more protective against coccidioidal infection. However, in spite of the promising results of immunization with rCsa+rAg2/Pra, there is a paucity of immunological data on the mechanisms of action of this combined recombinant vaccine in C57BL/6 mice, and concerns that it may not provide the same level of protection to a host with a Th2-biased response (e.g., BALB/c mice). It would be premature to claim that a vaccine against coccidioidomycosis is in hand. The benchmark of a successful vaccine has been established by the results of immunization of mice with the live, avirulent Δ*chs5* mutant strain; 100% survival of BALB/c mice beyond 45 days post-challenge, and sterility of their body organs. Significant progress has been made

toward the production of a coccidioidomycosis vaccine; we believe that generation of a successful, recombinant prophylactic human vaccine against this fungal pathogen is achievable.

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